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Identification of Transcription Factors *GZF3*, *RFX1*, Orf19.3928
as Being Implicated in *Candida*-Bacterial Interactions.

By

Joni M. Watson

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Abstract

Candida albicans is an opportunistic pathogen that is present in the normal flora in a majority of individuals. One key factor in *C. albicans* virulence is the ability to change its morphology from yeast to an elongated or hyphal form. The regulation of this morphogenesis relies in part upon quorum sensing (QS) molecules. *C. albicans* often exists as part of a mixed culture alongside other fungi and bacteria and is influenced by their presence as well as the presence of QS molecules that they produce. It has previously been demonstrated, using haploinsufficient mutants, that a library screen of *Candida albicans* can be utilized to identify genetic elements involved in response to bacteria.² In this study, a library of diploid homozygous transcriptional regulator knockout (TRKO) mutants were screened to identify strains capable of forming hyphae in the presence of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli*. We identified three strains that showed increased hyphae development compared to wild type *C. albicans*. The strains identified had deletions of the transcriptional regulating genes Orf19.3928, Orf19.2842 (*GZF3*), and Orf19.3865 (*RFX1*).⁴ These strains were tested for alterations of filamentation in liquid media, and biofilm formation. All three strains showed increased rates of biofilm formation compared to the wild type. Orf19.3928 showed altered response to farnesol, a marked in biofilm formation and no inhibition of filamentation when farnesol was present in liquid media. The *GZF3* deletion strain showed enhanced filamentation with all three bacterial

species while the *RFX1* deletion strain showed increased filamentation only with *E. coli* and *S. aureus*. In spent media, *GZF3* showed slight increases in filamentation in *E. coli* and *S. aureus* while *RFX1* had moderate increases in filamentation in *E. coli* and *S. aureus* and slight increases with *P. aeruginosa*.

Introduction

Candida species are eukaryotic, unicellular yeast, in the Family Saccharomycetaceae. They are commonly isolated from humans and often exists as a part of our natural flora in the mouth, gastrointestinal tract, and female genitourinary tract.¹⁵ In recent years it has been reported that *Candida* species are the fourth most common nosocomial infection⁸, which may be in part due to the rise in the use of broad spectrum antibiotics.^{23,15} Many species of *Candida* are not pathogenic, however several are able to cause disease in humans (Table 1). One species, *C. albicans*, causes the majority of infections and may be present in the oral cavity of up to 75% of the population.¹³

Candida that exists as part of our natural flora usually does not cause disease due to competition with the many other commensal organisms that exist in our microbiome. The use of broad spectrum antibiotics, while effectively eliminating the desired target organism responsible for disease, may also unintentionally eliminate aspects of our natural flora that help to control the levels of *C. albicans*.^{23,15} This can lead to overgrowth of *C. albicans* which can then establish an opportunistic infection. These opportunistic infections are seen more often in nosocomial settings and in immune compromised individuals such as premature infants, or those with AIDS, or diabetes.⁸

Candidiasis

Candida infections can be separated into two categories: superficial infections and systemic infections. Superficial infections are usually mild and self-limiting and are often seen in the mucous membranes of the mouth or genitourinary tract, causing

thrush and vulvovaginal candidiasis.¹³ The less common systemic infections, or candidemia, are much more severe and are more likely to occur in hospital settings due to invasive procedures such as catheter placement.⁷ Systemic infections are also more common in those with compromised immune systems, although individuals with normal immune responses can also develop *Candida* infections. Candidemia mortality rates have been reported to be more than 35%, with *C. albicans* species causing more than 45-68% of all candidemia.^{7,17} The ability of *C. albicans* to cause disease in such a wide spectrum of the population makes it important to understand factors that contribute to its virulence.

Species	Frequency	Morphology	Ploidy
<i>C. albicans</i>	68%	Yeast, Pseudohyphae, Hyphae	Diploid
<i>C. glabrata</i>	11.3%	Yeast, Pseudohyphae	Haploid
<i>C. tropicalis</i>	7.2%	Yeast, Pseudohyphae, Hyphae	Diploid
<i>C. parapsilosis</i>	6.0%	Yeast, Pseudohyphae	Diploid
<i>C. krusei</i>	2.4%	Yeast, Pseudohyphae	Diploid
<i>C. guilliermondii</i>	0.7%	Yeast, Pseudohyphae	Haploid
<i>C. lusitaniae</i>	0.6%	Yeast, Pseudohyphae	Haploid
<i>C. kefyr</i>	0.5%	Yeast, Pseudohyphae	ND*
<i>C. famata</i>	0.2%	Yeast, Pseudohyphae	Haploid
<i>C. inconscipua</i>	0.2%	Yeast, Pseudohyphae	ND*
<i>C. rugose</i>	0.2%	Yeast, Pseudohyphae	Haploid
<i>C. dubliniensis</i>	0.2%	Yeast, Pseudohyphae, Hyphae	Diploid
<i>C. norvegensis</i>	0.1%	Yeast, Pseudohyphae	ND*

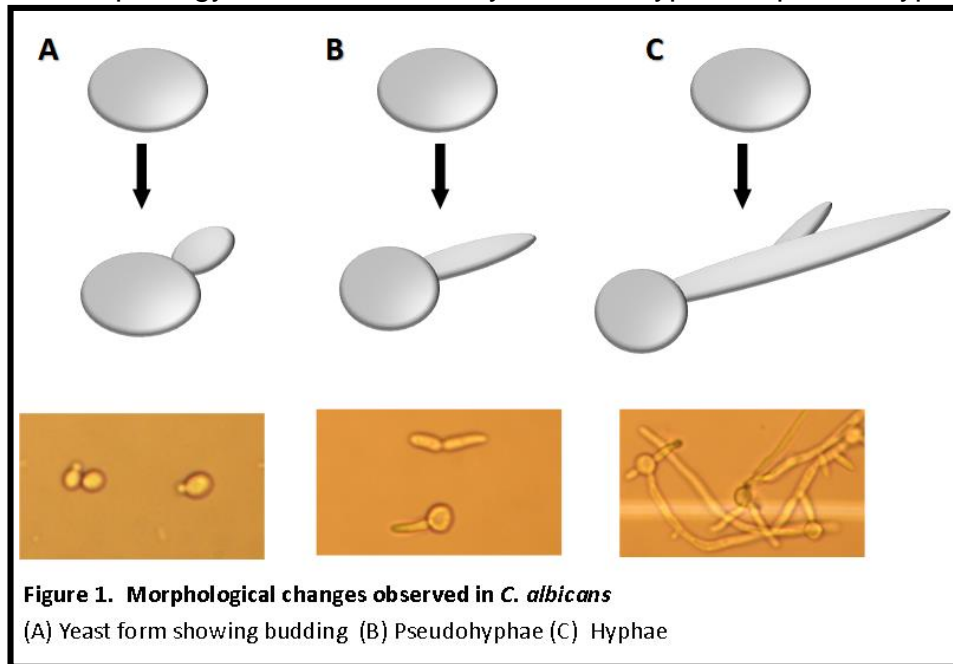
Table 1. Pathogenic *Candida* species.¹⁷

Candida species, their frequency observed as human pathogens, possible morphologies, and genetic organization.

*--Not Determined

Virulence

In order to cause disease *C. albicans* relies upon several virulence factors including adhesins, invasins, secreted proteases and hydrolytic enzymes.¹³ Another important virulence trait that *C. albicans* possesses is the ability to undergo a change in its morphology from a unicellular yeast to a hyphal or pseudohyphal form in response to



environmental changes (Figure 1). There are a number of environmental conditions that have been found to induce hyphal formation including starvation, the

presence of serum or N-acetylglucosamine, increased CO₂ levels, and pH above 7.¹³ Morphogenesis is also controlled by the presence of quorum sensing molecules.⁸ This polymorphism is key to the organism's virulence and it has been shown that when *C. albicans* is "locked in" to either the yeast or hyphal forms by genetic manipulation that its virulence is decreased.⁸ However, recent evidence has also shown that hyphal formation is co-regulated along with other virulence factors, so it is difficult to say for certain which factor is causing the decrease virulence.¹⁹

Hyphae or pseudohyphae contribute to virulence by invading the host tissues and destroying macrophages.¹⁵ Another important virulence factor is the ability of *C.*

albicans to form biofilms on the tissues of the host organism or on implanted medical devices.^{8, 12} Biofilms are microbial communities that form on either natural or abiotic surfaces.²¹ They have organized structures and can consist of either a single microbial species or a mixture of microbes surrounded by an extracellular matrix.²¹ Cells that are able to form biofilms often show increased resistance to environmental stress, antimicrobial agents, and host immune responses.¹³ *C. albicans* biofilms consist of both the yeast and hyphal forms of the organism as well as an extracellular biofilm matrix composed mainly of β 1,3-glucan (Figure 2).^{8,13} In many situations *C. albicans* forms mixed biofilms with other potential pathogenic organisms and is often co-isolated along with these bacteria in nosocomial settings, especially from implanted medical devices.¹²

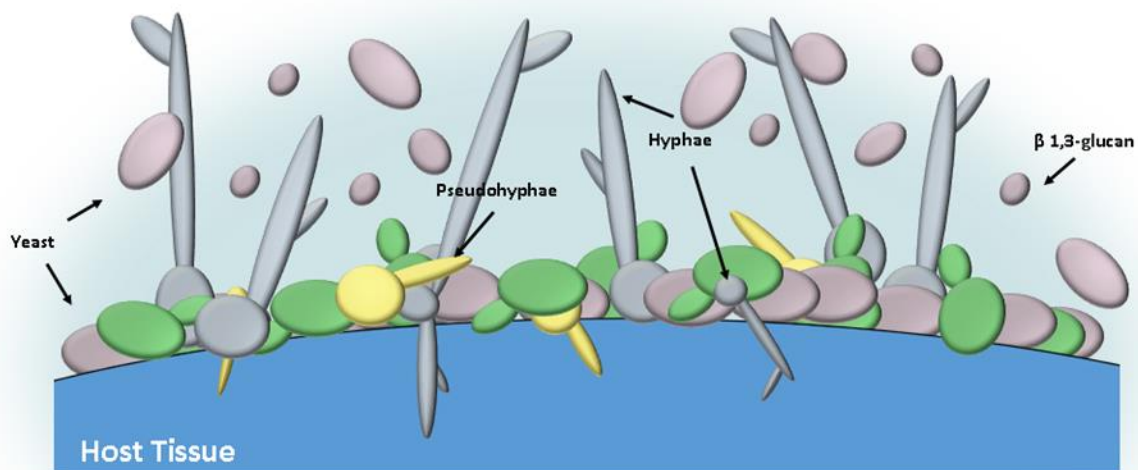
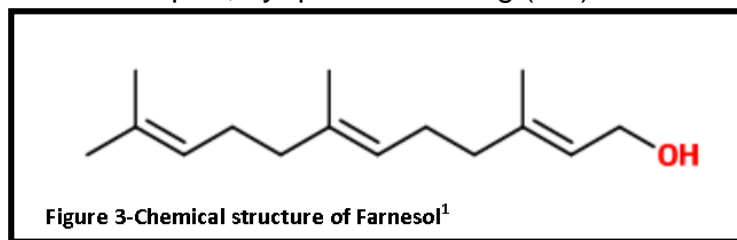


Figure 2. *Candida albicans* biofilm.

Biofilm formation showing composition including yeast, pseudohyphal, and hyphal morphologies, host tissue penetration by hyphal and pseudohyphal cells, and dissemination by planktonic yeast cells.

Quorum Sensing

The control of *C. albicans* morphogenesis has been found to be affected, at least in part, by quorum sensing (QS) molecules. There are several possible chemicals



that have been identified as possible QS molecules in *C. albicans* including phenylethyl alcohol, tryptophol,

morphogenic autoregulatory substance (MARS), tyrosol, and farnesol.⁸ Of these compounds, farnesol (Figure 3) seems to play one of the most crucial roles and is responsible for inhibiting hyphal formation, and much research has been conducted to determine its mechanism of action. As cell density increases, the amounts of farnesol eventually reach a threshold at which the yeast-hyphal transition is inhibited.^{8, 13}

Farnesol levels also play a vital role in the ability of *C. albicans* to form biofilms through its control of hyphal initiation. Cells grown at high farnesol concentrations show less attachment to surfaces and lower levels of biofilm formation.²⁰ Farnesol may, however, increase disseminated infection by leading to the release of planktonic yeast cells from a mature biofilm.²⁰ Farnesol has also been shown to have strong antibacterial and antifungal properties and likely plays a role in cross-kingdom interactions.¹

Candida-Bacterial Interactions

C. albicans often forms mixed biofilms and is co-isolated with other microorganisms. It is also estimated that >20% of bloodstream infections of *Candida* also involve a secondary bacterial infectious agent; therefore, the interactions that occur with other microbes must also be considered.¹⁹ Some of these interactions between microorganisms appear to be mutualistic while others can be antagonistic. In some

instances direct cell contact is needed to influence the other organism, while in others they affect each other indirectly, through competition for nutrients, or by secretion of molecules such as toxins or QS molecules.¹⁹ Many species of bacteria have been shown to inhibit filamentation by the production of QS molecules including:

Pseudomonas aeruginosa, *Lactobacillus rhamnosus*, *Saccharomyces boulardii*, *Burkholderia cenocepacia*, *Xanthomonas campestris*, *Acinetobacter baumannii*, *Salmonella enterica* Sero var Typhimurium, and *Streptococcus mutans*.¹⁹

Pseudomonas aeruginosa is often co-isolated with *C. albicans* from the lungs of cystic fibrosis patients, the wounds of burn victims, and from catheter-related biofilms.¹⁹ Hyphal formation is inhibited by QS molecules 3-oxo-C12 homoserine lactone (HSL) and dodecanol produced by *Pseudomonas*.^{5,19} *Pseudomonas* has also been shown to destroy hyphae by forming a biofilm on the hyphal filaments, restricting their growth and causing death of the hyphal cell.^{5,16} It has also been found that *C. albicans* has an antagonistic effect on *P. aeruginosa*. Farnesol, the secreted QS molecule produced by *C. albicans* inhibits swarming motility and impacts the synthesis of PQS in *P. aeruginosa* which is needed for the synthesis of pyocyanin, an importance virulence factor that is toxic to *C. albicans*.^{10,21} (Figure 4). This dual relationship likely is reflective of the fact that farnesol concentrations increase with cell density, so inhibition of PQS is probably not seen in many infections because the cell concentration is initially low.

In oral bacteria it has been shown that the production of *trans*-2-decenoic acid produced by *S. mutans* inhibits filamentation while AI-2 produced by *S. gordonii* stimulates filamentation and biofilm production.¹⁹ Another organism that has been shown to alter the morphology of *C. albicans* is *E. coli*. It has been reported that the

presence of *E. coli* lipopolysaccharide (LPS) is detected directly by *C. albicans* and results in an increase in hyphae formation, contributing to *C. albicans* virulence.^{12,17} It has also been reported that prior colonization with *E. coli* enhances adhesion of *C. albicans* to bladder mucosa.^{16,21}

In laboratory settings, *E. coli* appears to inhibit hyphal formation in growing *C. albicans* colonies, as do the other bacterial species *S. aureus* and *P. aeruginosa*.² The degree of inhibition varies among these different species and the mechanisms involved are still not completely understood. Better understanding of these cross- kingdom interactions is important and may lead to new ways to prevent or treat these infections.

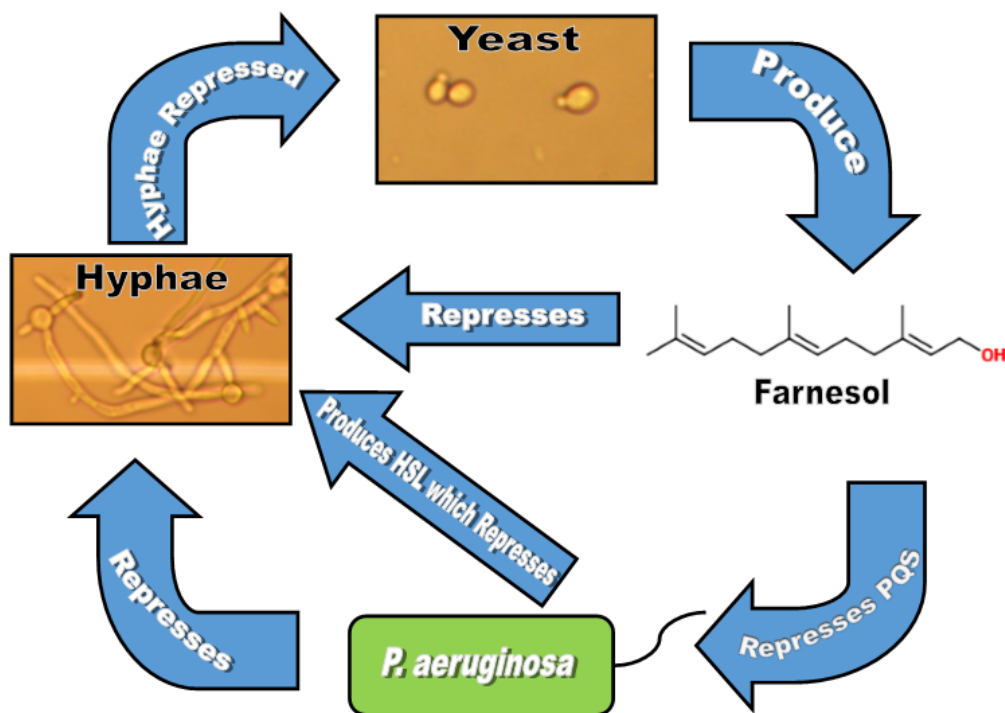


Figure 4. *P. aeruginosa* and *C. albicans* interactions.

Complex interactions seen between *P. aeruginosa* and *C. albicans* involving QSMs

Recently, research has been conducted in order to identify genes involved in cross-kingdom interactions between *C. albicans* and bacterial species. In a study by Fox et al., a haploinsufficient *C. albicans* mutant library was screened to identify genes involved in the regulation of hyphae production in the presence of *P. aeruginosa*, *Staphylococcus aureus*, and *E. coli*.² In this current study, a deletion library of *C. albicans* genes involved in transcriptional regulation was screened in order to identify mutants capable of producing hyphae in the presence of the same bacterial strains. These three bacterial strains were chosen because they are often co-isolated along with *Candida albicans* and identification of genes with altered morphologies in the presence of all three bacteria would more likely indicate that the gene is involved in a global response to bacteria. Colony morphology was examined to identify genes causing alterations in *C. albicans* morphology resulting in abnormal colony appearance. The genes identified were subjected to filamentation tests in liquid media, and biofilm formation assays to investigate how these genes influenced interactions between *C. albicans* and the bacterial species.

Materials and Methods

Strains

Diploid *C. albicans* transcriptional regulator knockout mutants (TRKO) constructed by Homann, et. al.⁶ were obtained from the Fungal Genetics Stock Center.¹⁴ Freezer stocks were inoculated into liquid YPD and grown overnight for further testing. Wild type *C. albicans* SC5314 was used as a control microorganism. The three bacterial strains used were *E. coli* (ATTC#33922), *P. aeruginosa* (ATTC27853), and *S. aureus* (ATTC#25923).

Media, and growth conditions

The yeast strains were cultured on Yeast Peptone Dextrose (YPD) media (20g dextrose, 20g peptone, 10g yeast extract, 20g bacto agar in 1L dH₂O) at 30° C overnight. The bacterial strains were maintained on Luria Broth (LB) medium (10g tryptone, 10g NaCl, 5g yeast extract, 20g bacto agar in 1L dH₂O) at 37°C. For filamentation testing Medium 199 (9.5g M199, 18.7g Tris Buffer, 18g bacto agar, 25 mg Arginine, pH7.5 in 1L dH₂O) was utilized. Farnesol was prepared weekly in ethanol and was added to M199 at a 300 µM concentration.

Screen for filamentous mutants

The library of TRKO mutants were individually spot replicated with two centimeter spacing onto YPD, M199, or M199 plates pre-coated with bacterial lawns from fresh culture grown overnight at 37° C with shaking. The YPD plate served as a positive

control for growth and negative control for filamentation and was grown at 30° C for 48 hours. The M199 plates were incubated at 37° C and were observed at 5-7 days. The plates were observed under light microscopy for filamentation. The screen was repeated twice and knockouts that consistently showed filamentation with all three bacterial species were identified.

Filamentation in liquid media

The TRKO mutants identified as filamentous with bacteria in the library screen were tested for filamentation in liquid media. The strains were grown aerobically overnight in liquid YPD at 30° C with shaking, harvested by centrifugation, washed three times with sterile dH₂O and counted with a hemocytometer. Pre-warmed M199 was inoculated with 5x10⁵ cells/mL with four conditions: M199 with no bacteria, and M199 with overnight growth of *E. coli*, *S. aureus*, and *P. aeruginosa*. The *C. albicans* strains were incubated for 3 hours at 37° C, were centrifuged to concentrate the cells, and a morphology assessment was performed with a light microscope. The filamentation assay was also performed utilizing spent media. M199 containing overnight bacterial growth were centrifuged for 8 minutes at 8,000x g and the media was then aspirated, leaving pelleted bacteria. Centrifugation was then repeated, along with aspiration, to ensure removal of the maximum amount of bacterial cells. The spent media was then inoculated with the *C. albicans* strains at 5x10⁵ cells/mL and were incubated for 3 hours at 37° C and were observed for cell morphology. A filamentation assay was also performed with 300 µM Farnesol present in the M199. Farnesol was added to the pre-warmed M199 media and was then inoculated with *C. albicans* strains at 5x10⁵

cells/mL. These cultures were incubated at 37° C for 3 hours and morphology was assessed.

Biofilm formation

To assess for biofilm formation the method described by Peeters, et. al. was utilized.¹⁸ Briefly, a 96 well plate was prepared by placing 100µL 5% Fetal Bovine Serum (FBS) into each well and was refrigerated overnight. The yeast to be tested was inoculated into liquid YPD and was grown overnight at 30° C with shaking. The cells were isolated via centrifugation, washed three times with sterile water and counted with a hemocytometer. A serial dilution of the cells (1×10^5 , 1×10^4 , 1×10^3 , 1×10^2) was then prepared in pre-warmed M199.

After removal of the FBS, the diluted cells were placed into the 96 well plate in triplicate and were incubated at 37°C for 90 min to allow cells to adhere. At the end of the 90 minute period the M199 was removed and the wells were gently washed with 100µL sterile water to release any unadhered cells, then 150µL of sterile, pre-warm M-199 was added to each well. The plates were wrapped with parafilm and incubated at 37°C for 48 hours. At the end of the 48 hour period the M199 was removed from the 96 well plate with a vacuum tip. In order to remove any unadhered, planktonic cells each well was gently washed with 100µL sterile water. The cells were then fixed by adding 100µL ethanol to each well for 10 minutes. After 10 minutes the ethanol was removed with the vacuum tip and the plate was allowed to air dry for 20 minutes. The remaining adhered cells in the biofilm were stained with 0.0076% crystal violet for 15 min. The stain was removed with vacuum tip. 150µL 33% acetic acid was placed into each well and absorbance was measured at 590 nm. This biofilm procedure was repeated to test

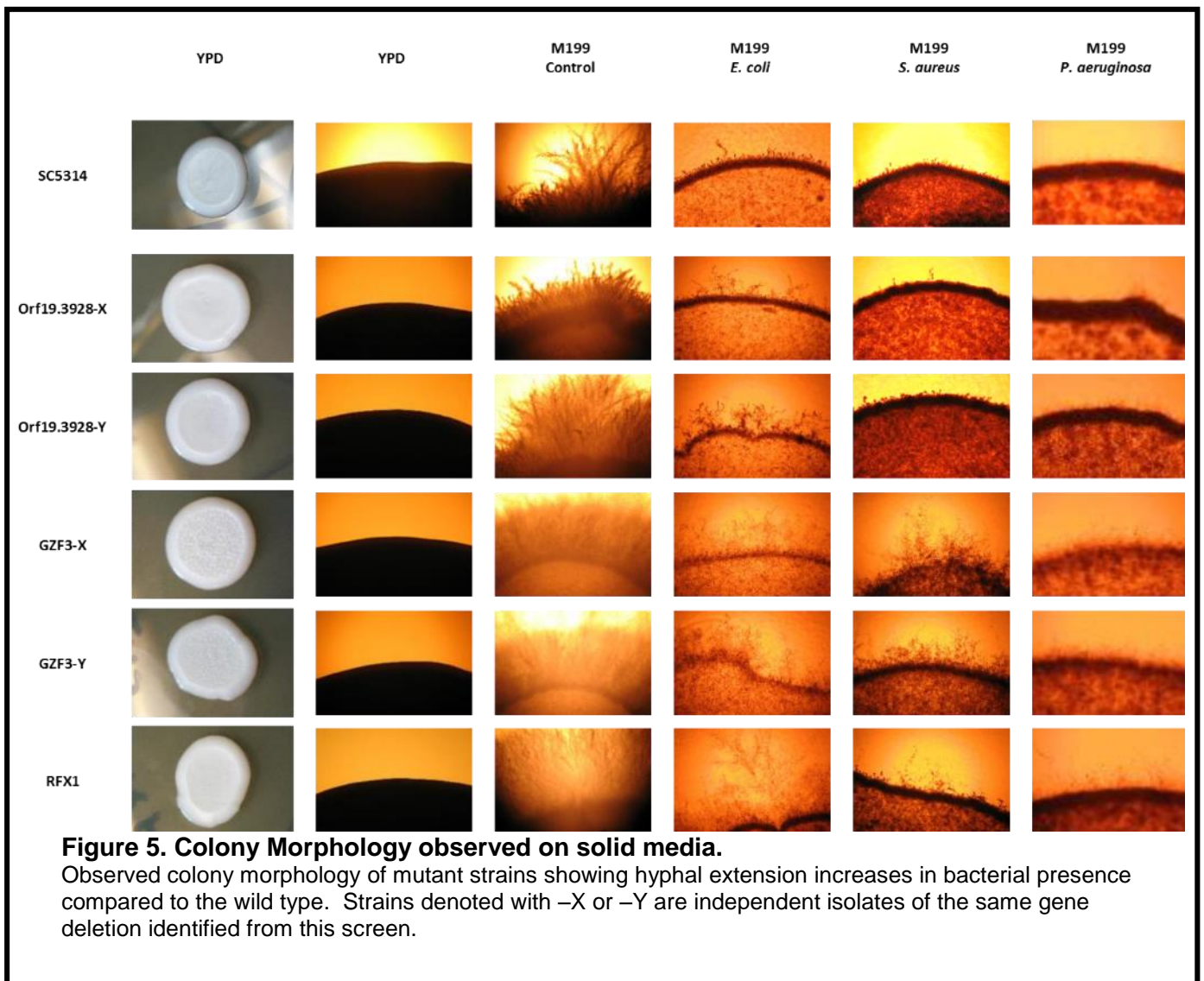
biofilm formation in the presence of farnesol by adding pre-warmed M199 containing 300 μ M farnesol to the wells following cell adhesion.

Results

Identification of three transcriptional regulators by a homozygous deletion screen.

In order to identify genetic elements involved in response to the presence of the bacteria or their secreted products we first performed a library screen of the TRKO mutants. The library and the control (SC5314) strains were individually spot replicated onto YPD, M199, and M199 with bacterial lawns. The bacterial strains chosen included both Gram positive and Gram negative organisms known to share similar host environments to *C. albicans*. The fresh lawns on the M199 plates established a competitive environment with the *C. albicans* and would resemble interactions of the host environment. The YPD plate served as a negative control for filamentation and was incubated at 30° C, those strains that were observed to be filamentous after 48 hours were removed from the screen. The M199 plate at 37° C was used as a control for filamentation and those strains that did not show filamentation after 5-7 days were eliminated from the screen. In total, 364 strains were evaluated and we identified 5 (1.4%) strains as potential candidates for further study as being filamentous in the presence of all three bacterial species. Four of the strains consisted of duplicate deletions of identical genes (Orf19.3928, *GZF3*) while the fifth was a gene for which a second independent transcription factor deletion was not available (*RFX1*). Figure 5 shows representative colony morphologies seen in the YPD control as well as on M199 for both wild type organisms and those found to be filamentous with all three bacteria.

On YPD at 30° C the wild type organisms show typical yeast-type colony morphology with smooth rounded edges. In contrast, the M199 plate at 37° C shows hyphae extending from the colony into the surrounding media. In the presence of bacteria, hyphal formation is inhibited in the wild type strains and the colonies lack hyphal filaments protruding from the colonies. The TRKO strains identified in our screen exhibited hyphal growth in the presence of all three bacterial species.



Filamentation in Liquid media

In addition to the ability to filament on solid media, we tested the ability of the genetic mutants to filament in liquid media. *C. albicans* mutants were grown overnight and inoculated at 5×10^5 concentration into M199 at 37°C to induce hyphae formation. The TRKO mutants were tested in M199 in three conditions: M199 with overnight bacterial growth of *P. aeruginosa*, *S. aureus*, and *E. coli*; Spent M199 (overnight bacterial growth with cells removed); and M199 with 300 μ M farnesol. We found that the gene deletion of Orf19.3928 showed enhanced filamentation in liquid when cultured alongside *E. coli* and *S. aureus*, but showed filamentation rates similar to the wild type when cultured with *P. aeruginosa*. The *GZF3* deletion strain showed enhanced filamentation with all three bacterial species while the *RFX1* deletion strain showed increased filamentation only with *E. coli* and *S. aureus*. (Figure 6) In spent media, *GZF3* showed slight increases in filamentation in *E. coli* and *S. aureus* while *RFX1* had moderate increases in filamentation in *E. coli* and *S. aureus* and slight increases with *P. aeruginosa*. (Figure 7) When exposed to farnesol, *GZF3* still displayed hyphae formation while *RFX1*, Orf19.3928 and the wild type did not.

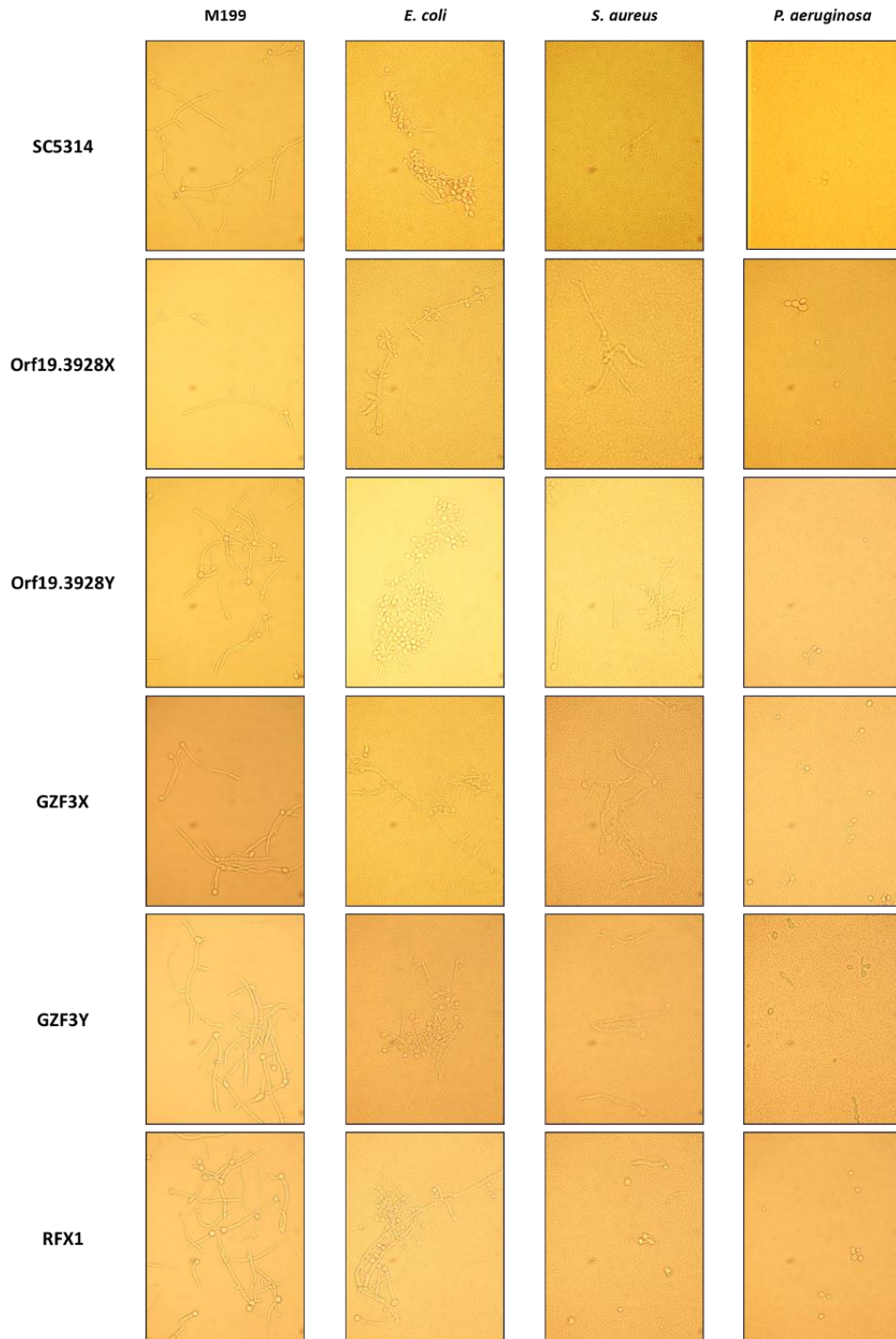


Figure 6. Filamentation in the presence of bacteria in liquid media.

Increased in filamentation rates in deletion strains when compared to the wild type. Strains denoted with -X or -Y are independent isolates of the same gene deletion identified from this screen.

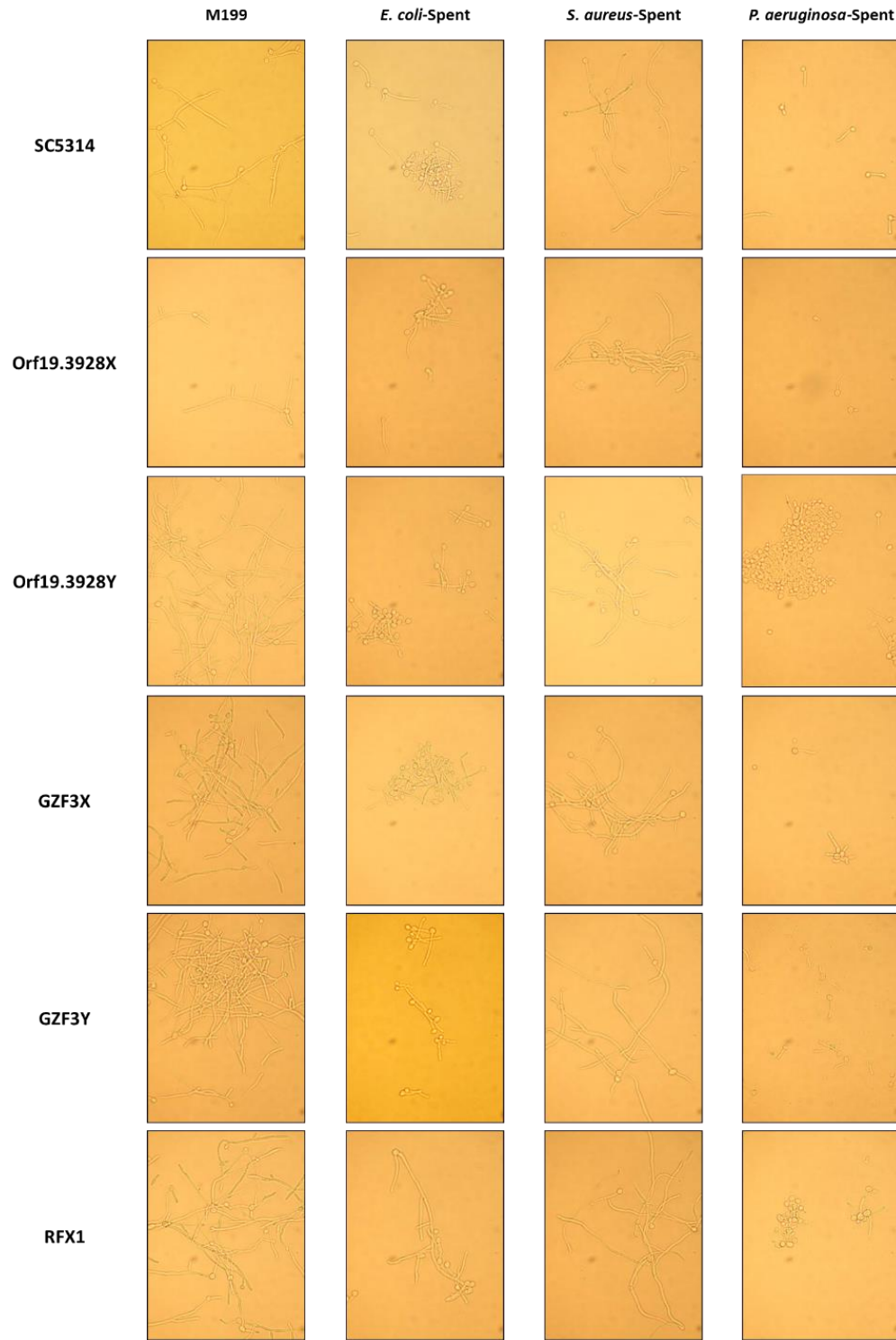


Figure 7. Filamentation in spent media.

Some increases in filamentation rates in deletion strains when compared to the wild type. Strains denoted with -X or -Y are independent isolates of the same gene deletion identified from this screen.

Biofilm Formation

We were able to determine the ability of each TRKO strain to form biofilms in M199 with and without the presence of farnesol over a 48 hour period. Following growth the biofilms were washed, fixed, and stained. Optical Density (OD) analysis was performed at 560 nm and we found that all of the strains identified showed increased biofilm formation with and without the presence of farnesol. *RFX1* showed a marked increase over the other strains when farnesol was not present, but was reduced greatly with the addition of farnesol. While in the presence of farnesol Orf19.3928 showed the highest biofilm formation, which is in agreement with the finding that filamentation was seen in liquid media with farnesol. (Figure 8, 9)

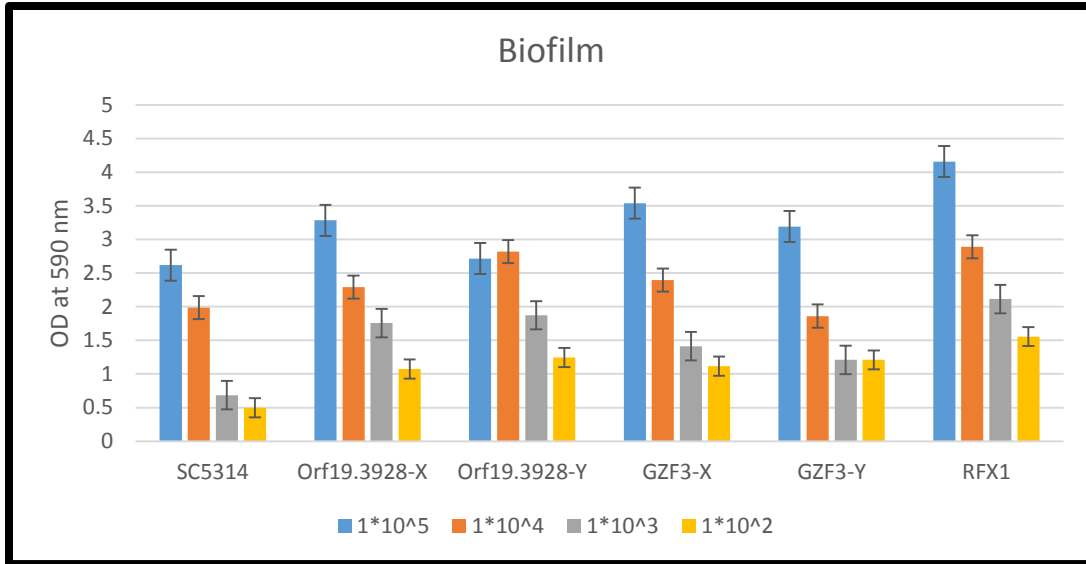


Figure 8. Biofilm formation without farnesol.

Biofilm formation of mutants compared to wild type, showing increased formation by all mutant strains. Strains denoted with –X or –Y are independent isolates of the same gene deletion identified from this screen.

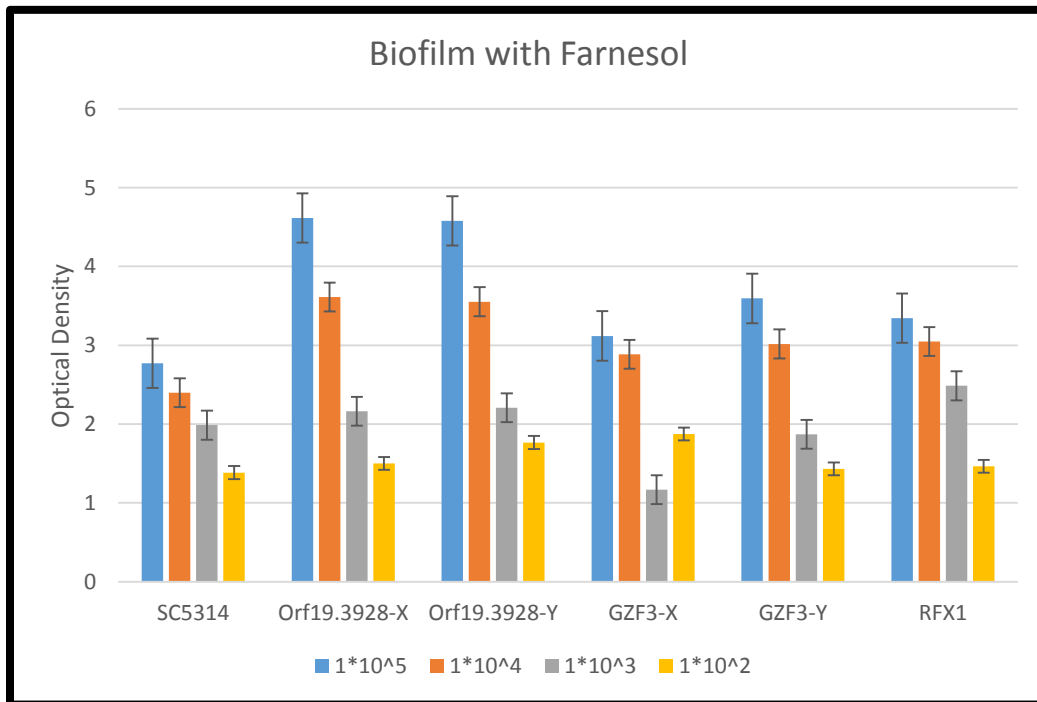


Figure 9. Biofilm formation in the presence of farnesol.

Biofilm formation of mutants compared to wild type, showing increased formation with mutant strains, especially Orf19.3928. Strains denoted with –X or –Y are independent isolates of the same gene deletion identified from this screen.

Discussion

In recent years, research has revealed how important the microbiome that exists within our body is to maintain normal function and prevent disease. Imbalances in our microbiome have been shown to lead to decreases in our natural immunity and increased risk of infection. The natural flora of our bodies not only provides a preventative measure against disease but restoring the balance of microbes in our flora can also be used to treat some infections, such as *C. difficile*.³ A large part of understanding how our microbiome works to our benefit as well as contributes to disease is understanding how all of the organisms that exist in our microbiome interact with each other and with us. The conversation between these organisms is complex and we are only beginning to understand a part of the communication that occurs.

Candida albicans is a part of the normal flora and normally its growth is restricted by the presence of other microbial species or their secreted products. When patients are treated with broad spectrum antibiotics or are immune compromised these factors are diminished, which can lead to the establishment of an opportunistic *C. albicans* infection.

In *C. albicans*, it has been demonstrated that quorum sensing plays a role not only in communication from within the species but also for interspecies communication. Several genes have been identified to regulate filamentation in *C. albicans* such as *TUP1*, *CPH1* and *EFG1*.¹ In addition, genes have also been discovered that play a role in quorum sensing (QS) in *C. albicans*. For example, the gene *CHK1* has been

identified as a key component in the response of the self-regulating QS molecule farnesol.⁹ The null mutant (*chk1/chk1*) and the revertant mutant (*chk1/CHK1*) both are able to filament in the presence of farnesol at low cell densities, while the wild type strain was inhibited by the farnesol.⁹

While the mechanisms of hyphal formation and quorum sensing have been studied in depth, relatively little is known about the genetic factors that play a role in *C. albicans* interactions with bacteria. Fox et. al. screened a haploinsufficient transposon insertion library of over 18,000 mutants and identified 107 known/predicted genes that were involved in *Candida albicans*/bacterial interactions. The genes fell into several categories including genes related to enzymatic activity, transport, transcription, signaling, and adhesion.²

The library screen completed in this study was much narrower in scope and utilized a library of diploid organisms consisting of only transcriptional regulator deletion mutants. In this study we identified three total genes involved in bacterial interactions. The gene located at Orf19.3928 is uncharacterized as of yet. The second gene Orf19.3865 has been identified as *RFX1*, a gene involved in DNA repair.⁴ Hao et. al., demonstrated that deletion of *RFX2*, a similar gene also involved in DNA repair, resulted in a mutant that was significantly more resistant to environmental stresses such as UV irradiation, heat shock, and ethanol.¹¹ The third gene Orf19.2842 has been identified as *GZF3*, which encodes for a GATA-type transcription factor induced during oxidative stress.²² A common theme among the named genes that we identified in this study is that they have been found to be involved in response to stressful environments. Bacteria living in mixed microbial communities often secrete factors such as acidic

products or reactive oxygen species to impair the growth of other microbes. Recent research has also shown that there is a possible link between quorum sensing and oxidative stress resistance, with farnesol providing protection against H₂O₂ and superoxide anion-generating agents.²⁴ It is unclear how these genes identified may help protect *C. albicans* from bacterial competition or if they may be involved in response to QS molecules. More research is needed to determine the possible mechanisms behind these interactions so that we can better understand how *C. albicans* communicates and regulates its morphology in mixed microbial communities.

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